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A novel interplay between the Fanconi anemia core complex and ATR-ATRIP kinase during DNA cross-link repair

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ABSTRACT

When DNA replication is stalled at sites of DNA damage, a cascade of responses is activated in the cell to halt cell cycle progression and promote DNA repair. A pathway initiated by the kinase Ataxia teleangiectasia and Rad3 related (ATR) and its partner ATR interacting protein (ATRIP) plays an important role in this response. The Fanconi anemia (FA) pathway is also activated following genomic stress, and defects in this pathway cause a cancer-prone hematologic disorder in humans. Little is known about how these two pathways are coordinated. We report here that following cellular exposure to DNA cross-linking damage, the FA core complex enhances binding and localization of ATRIP within damaged chromatin. In cells lacking the core complex, ATR-mediated phosphorylation of two functional response targets, ATRIP and FANCI, is defective. We also provide evidence that the canonical ATR activation pathway involving RAD17 and TOPBP1 is largely dispensable for the FA pathway activation. Indeed DT40 mutant cells lacking both RAD17 and FANCD2 were synergistically more sensitive to cisplatin compared with either single mutant. Collectively, these data reveal new aspects of the interplay between regulation of ATR-ATRIP kinase and activation of the FA pathway.

INTRODUCTION

Fanconi anemia (FA) is a hereditary disorder characterized by cancer susceptibility and hypersensitivity to inducers of DNA interstrand cross-links (ICLs) (1,2). FA is caused by mutations in a genetically and biochemically complex set of proteins, including an FA core E3 ligase complex containing eight FA gene products (i.e. FANCA, B, C, E, F, G, L, M) and other associated proteins (i.e. FAAP100, FAAP24, FAAP20) (1,2). The FANCM–FAAP24 subcomplex is thought to load the

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rest of the core complex onto damaged chromatin (3,4). The core complex mediates monoubiquitination of the ID complex composed with FANCD2 and FANCI proteins. The monoubiquitinated ID complex in turn recruits the DNA repair nuclease FAN1 (2,5–7), and might function as histone chaperone during ICL repair (8). In addition, it has been suggested that the core complex might have other functions (9). Specific mutations of some additional FA genes (*FANCD1/BRCA2*, *FANCJ/BRIP1*, *FANCN/PALB2*, *FANCO/RAD51C*) are found in familial breast cancer (2,10–12), but these genes and a novel FA gene *FANCP/SLX4* (13,14) do not affect the core signaling pathway, resulting in monoubiquitination of the ID complex. Slx4 is shown to be recruited by monoubiquitinated FANCD2 (15) and contributes to ICL repair mainly through regulation of XPF-ERCC1 nuclease (16).

A critical DNA damage response pathway is mediated by the checkpoint kinase ATR and its protein partner ATRIP. One connection between the FA pathway and ATRIP has been uncovered previously: the checkpoint kinase ATR-ATRIP controls multiple phosphorylation events on FANCI, which trigger FA pathway activation (17–20). ATR kinase activation proceeds in two largely independent steps (21–23): first, a stalled DNA replication fork generates a stretch of single-stranded DNA (ssDNA) covered by Replication protein A (RPA) complex, which in turn recruits ATRIP-ATR into distinct focal areas within cell nuclei. Interaction of RPA-bound ATRIP-ATR with the TOPBP1 protein leads to execution of the S-phase checkpoints. The latter step also involves the specialized RAD9-RAD1-HUS1 (9-1-1) checkpoint clamp and the RAD17-RFC clamp loader (21–23), but the molecular details of these processes are unclear.

We wished to clarify how ATR signaling and the FA pathway are coordinated. We examined the ATR signaling events in FA cell lines, and found that the FA core complex does not simply lie downstream of ATR, but functions in ATR kinase activation after replication stress by enhancing chromatin binding of ATRIP. Unexpectedly, we also found that the canonical ATR activation pathway involving RAD17 and TOPBP1 is largely dispensable for activation of the FA pathway. Taken together, our current data provide novel insights regarding the interplay between ATR-ATRIP kinase and activation of the FA pathway.

MATERIALS AND METHODS

Cell culture, gene targeting and transfection in DT40 cells

Wild-type (WT) and various mutant chicken DT40 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 1% chicken serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and penicillin/streptomycin in a 5% CO_2 incubator at 39.5°C. Generation of *rad17*, *atm*, *fancd2*, *fancc*, *fancl*; *fancl* complemented with Green fluorescence protein (GFP)-chFANCL cell lines has been described previously (24–28). *FANCM*-deficient DT40 cells (29), *FANCM* D203A 'knock-in' cells (30), *usp1* (31) and *ube2t* knockout (32) cell lines were kindly provided by Dr K.J. Patel (Cambridge University).

Full-length chicken ATRIP cDNA was amplified by reverse transcriptase polymerase chain reaction (PCR) from DT40 RNA and cloned into pDONR vector (Invitrogen). After sequencing, the gateway system (Invitrogen) was used to transfer the cDNAs to the GFP expression vector (20). Targeting vectors were constructed by subcloning PCR-amplified genomic fragments on both sides of the resistance gene cassettes. All transfections in DT40 were done as described (17).

FANCI K525R 'knock-in' was achieved in a heterozygous *FANCI* knockout clone (17), and the resistance gene cassette flanked by the flippase recognition target (FRT) sites introduced into intron was removed by flippase (FLP) recombinase-mediated excision (Flp expression plasmid was provided by Dr Kyoji Horie, Osaka University). Briefly, cells were transiently transfected with a plasmid encoding FLP recombinase and IRES-puro, and excised cells were isolated by puromycin selection for 2 days followed by limiting dilution. While *fanci*-K525R cells could not monoubiquitinate FANCI, they showed a partial defect in FANCD2 monoubiquitination on DNA damage by mitomycin C (MMC), and displayed only mild sensitivity to cisplatin compared with *fanci* null cells. Conditional *FANCD2* knockout cells were made by knock-in targeting of two FRT sites in *FANCD2* heterozygous knockout clone. The FRT sites flanked a genomic segment that included the exon containing the monoubiquitination site. The resistance gene cassettes were removed by expression of Cre recombinase using plasmid-based transfection. In this conditional cell line, *FANCD2* gene could be inactivated by FLP-mediated excision. *RAD17* gene was targeted in the conditional *FANCD2* knockout cells using RAD17 targeting vector previously described (25).

Human cell cultures and transfections

Human ATRIP-GFP or FANCL-Flag expression vector was constructed by cloning PCR product from HeLa cell cDNA into appropriate expression vectors. GFP-human FAAP100 was similarly made by PCR from a plasmid (a kind gift from Dr Weidong Wang).

Human HeLa S3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and penicillin/streptomycin in a 5% CO_2 incubator at 37°C, and were introduced with retroviral bicistronic expression vector encoding FLAG-HA-tagged human ATRIP (FLAG-HA-ATRIP) and IRES-CD25 as described (33). The retrovirus was packaged using 293P cells, which were maintained in DMEM supplemented with 10% newborn calf serum and penicillin/streptomycin. The transduced cells were selected on the basis of CD25 expression using Dynabeads (33).

GM6914 (FA-A) with or without complementation with FANCA (a gift from Dr Takayuki Yamashita, Gunma University), and PD20 cells with or without complementation with GFP-hFANCD2 (a gift from Dr Toshiyasu Taniguchi, Fred Hutchinson Cancer Center) were maintained in α-minimum essential medium

(MEM) supplemented with 20% FCS, 1% penicillin and streptomycin. Human ATRIP-GFP-expression vector was transfected into GM6914 and control cells using Lipofectamine LTX according to the manufacturer's instructions. A549 cells expressing ATRIP-GFP have been described (34), and maintained in DMEM with 10% FCS. siRNA transfections were done with Lipofectamine RNAiMAX. The messenger RNA target sequences used for siRNAs were as follows: Rad17 (GAUGGGUCAAC CCAGUCUGTT), TopBP1 (GUGGUUGUAACAGCG CAUC), FANCA (AAGGGUCAAGAGGGAAAA AUA), FANCD2 (CCAUGUCUGCUAAAGAGCGUU CAUU).

Antibodies

Anti-chicken FANCD2 (35) or anti-chicken FANCI (20) serum was described. Other antibodies were purchased from Santa Cruz Biotechnology (polyclonal anti-ATR: sc-1887, polyclonal anti-RAD17: sc-5613, monoclonal anti-Chk1: sc-8408), Bethyl Laboratories (polyclonal anti-FANCA: A301-980A, polyclonal anti-RPA32: A300-244A), Abcam (polyclonal anti-Histone H3: ab1791, polyclonal anti-FANCL: ab42639), Cell Signaling Technology (polyclonal anti-phospho-Chk1-Ser317: #2344), Novus (polyclonal anti-TopBP1: NB100-217, polyclonal human FANCD2: NB100-182, polyclonal anti-FANCG: NBP1-06035), Millipore (monoclonal anti-phospho-ATM-Ser1981: 05–740), Clontech (polyclonal anti-GFP: 632459), Sigma (monoclonal anti-tubulin: T5168) and Upstate Biotechnology (monoclonal anti-CD25: #05–170).

Fractionation of cells and western blotting

Cells were treated with indicated dose of DNA damaging agents and collected. Cell fractionation into soluble and chromatin fractions was done as described previously (36). Briefly, washed cells were resuspended in CSK buffer (10 mM PIPES, pH 7.0, 100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 10 mM NaF, 25 mM β-glycerophosphate, 0.2% Triton X-100, 0.25 mM ATP) containing protease inhibitor tablet (Roche), and centrifuged. Supernatants were saved as soluble fraction. Pellets were further washed using the same buffer twice, and the remained material was used as chromatin fraction. These samples were separated by polyacrylamide gel electrophoreses, transferred to a membrane and detected with indicated antibodies and ECL reagents (GE Healthcare). Phos-tag western blotting was done as described (17). Co-immunoprecipitation assay between overexpressed proteins in 293T cells was carried out as described (27).

Analysis of growth and cell sensitivity toward cisplatin

Cell proliferation rate was assessed as described using plastic microbeads. Cell viability in liquid culture containing cisplatin (Nippon Kayaku, Tokyo, Japan) was assessed after 48 h using FACSCalibur (BD Biosciences) and propidium iodide (PI) staining. Percentage of viable cells was calculated on the basis of forward scatter profile and exclusion of PI fluorescence among the acquired 10 000 events.

Subnuclear focus formation assay

After MMC (Kyowa-Hakkou-Kirin, Tokyo) exposure, cytospin slides or coverslips were fixed with 4% paraformaldehyde/phosphate buffered saline and stained with antibodies against FancD2 or RPA2. Then cells were stained with AlexaFluor-conjugated secondary antibody (Invitrogen) with 4′,6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) counterstaining. Images were captured by fluorescent microscopy (DM5500B, Leica) or confocal laser scanning microscopy (TCS SP5, Leica).

RPA-ssDNA pull-down assay

This assay was carried out essentially as described (37). In brief, biotin-labeled 75 mer ssDNA (5 pmole) was attached to streptavidin-coated magnetic beads (Dynal), and incubated with 400 ng of purified recombinant RPA for 30 min in binding buffer A (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 ng/ml bovine serum albumin (BSA), 0.01% Nonidet P-40, 10% glycerol). The RPA complex was purified as described (38,39) from *Escherichia coli* carrying the RPA-expressing plasmids kindly provided by Dr Marc S. Wold (University of Iowa College of Medicine, Iowa City, Iowa, USA). Cell lines were lysed in 220 µl of lysis buffer (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.05% Nonidet P-40, 10 ng/ml BSA, 10% glycerol). Resultant lysate (100 µl) containing equal amount of protein was then mixed with the beads, and incubated for additional 30 min at room temperature. After washing, the bound material was eluted by addition of sodium dodecyl sulphate-polyacrylamide gel electrophoresis sample buffer and boiling, and examined by western blotting.

Yeast two-hybrid assay

The yeast strain EGY48 (Clontech) was used as a host to express fusion proteins with LexA or B42 transcription activation domain fusion. cDNAs encoding ATRIP, and human FA and FA-related proteins (FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, UBE2T, FAAP24 or FAAP100) were cloned into pLexA (bait) and pB42AD (prey) vectors (Clontech). Sources of the most cDNAs were previously described (17,27,40). FANCM cDNA was provided by Dr Weidong Wang. Human UBE2T or FAAP24 cDNA was amplified from HeLa cell cDNA. The transformants were selected on SD/-Ura/-His/-Trp plate, cultured in liquid of the same medium at 30° for 24 h and then spotted on to SD/-Ura/-His/-Trp/-Leu/+X-gal plates at 30° for 96 h.

RESULTS

Defective localization of ATRIP following MMC damage in cells lacking the FA core complex

At sites of DNA damage and replication fork stress, ATRIP-ATR concentrates in nuclear foci mediated by binding of ATRIP to RPA-coated ssDNA (41). To examine potential interplay between ATR signaling and the FA core complex, we examined focus formation of

ATRIP following MMC-induced DNA damage in FA cells. Available anti-ATRIP antibodies did not allow convincing visualization of focus formation, and so we expressed a human ATRIP-GFP fusion in cells lacking FANCA. In MMC-untreated GFP-positive cells, ATRIP-GFP mostly distributed diffusely throughout the nuclei irrespective of the genotype. On MMC treatment, the complemented control cells displayed robust RPA foci formation that co-localized with ATRIP-GFP foci (Figure 1A). Strikingly, we found that ATRIP-GFP foci in MMC-stimulated FA-A cells were diffuse and greatly reduced, while RPA foci formed normally (Figure 1A). These data suggest that FANCA is required for MMC-induced ATRIP focus formation.

To further study ATRIP localization in the absence of FA proteins, we stably expressed chicken ATRIP-GFP in chicken DT40 WT and FA knockout cell lines. Similar levels of GFP expression among transfectants were confirmed with FACS analysis (Supplementary Figure S1A). Consistent with the results with human FA-A cells, MMC-induced chicken ATRIP-GFP focus formation was abrogated in *fancc* and *fancm* cells (Figure 1B). In contrast, extensive foci formation was still evident in *fancd2* cells, although observed foci were smaller and less intense (Figure 1B). The ATPase activity of the FANCM translocase domain was largely dispensable for focus formation, as shown in cells carrying a D203A knock-in mutation (Figure 1B) (30). The E3 ubiquitin ligase function of FANCL is unlikely to be important for chromatin localization of ATRIP because the E2 enzyme for FANCL, UBE2T, was dispensable for ATRIP focus formation (Figure 1B). The deubiquitinase for FANCD2, USP1, was also not required (Figure 1B). These data suggest that the FA core complex may play an important role in relocalization of ATRIP following DNA damage.

Decreased chromatin localization of ATRIP in cells deficient in the FA core complex

We next determined whether endogenous ATR and ATRIP show defective relocalization to chromatin in human FA cell lines. FA-A (GM6914) and FA-D2 (PD20) cells, with isogenic complemented controls, were treated with MMC, biochemically fractionated and analyzed by western blotting. Consistent with the foci results, FA-A cells displayed a significant reduction (~40%) in MMC-induced chromatin targeting of ATRIP relative to complemented control cells (Figure 2A). ATR localization in GM6914 cells may be deregulated because the ATR level in chromatin was increased in the untreated cells, and was not further increased following MMC treatment. In contrast, MMC-stimulated FA-D2 cells had only minor reduction of ATRIP-ATR levels in chromatin compared with the complemented control cells (Figure 2A). In line with the decreased chromatin binding of ATRIP, phosphorylation of ATRIP on Ser 68 and 72 was decreased to ~50% in FA-A cells but not in FA-D2 cells (Figure 2A and Supplementary Figure S2B and S2C). This phosphorylation event occurs in chromatin and is dependent on ATR (34).

To extend these observations to cells acutely rendered defective in FA proteins, we also examined HeLa S3 cells expressing FLAG-HA-ATRIP treated with siRNAs against FANCA or FANCD2. This cell line expresses ~2-fold more ectopically expressed tagged ATRIP than endogenous ATRIP. Cells depleted of FANCA reduced chromatin ATRIP levels by ~5-fold on MMC damage compared with control cells. The ATR level was also modestly decreased (Figure 2B). This could be partially accounted for by DNA damage-induced decrease in total ATR levels as shown in Figure 2C, though the reduction was greatest in cells exposed to control siRNA. On the other hand, cells depleted of FANCD2 did not have decreased chromatin loading of both ATRIP and ATR (Figure 2B).

Collectively, we conclude that the FA core complex component FANCA promotes or stabilizes chromatin localization of ATRIP in human cells. Combined with the ATRIP-GFP foci results in DT40 cells, the data suggested that the FA core complex as a whole functions to regulate ATRIP levels in chromatin.

The FA core complex is required for ATRIP localization upon replication stress

To address whether the FA core complex plays a similar role following other types of DNA damage, we treated cells with ionizing radiation (IR) or the replication stress inducers MMC, hydroxy Urea (HU) and UV. ATRIP was less phosphorylated on Ser 68/72 in FA-A cells than in isogenic control cells on replication stress, particularly following HU treatment, while the phosphorylation was not decreased in FA-A cells compared with control following IR (Supplementary Figure S3A). Furthermore, the ATRIP-GFP foci formation in *fancc* DT40 cells treated with HU was drastically reduced, while the same cells treated with IR showed similar levels of ATRIP-GFP foci formation to WT cells (Supplementary Figure S3B and S3C). These observations indicate that the dependence of ATRIP localization in chromatin on the FA core complex is dependent on the type of genomic stress. Stalled DNA replication forks (MMC, HU, UV) require the FA core complex for ATRIP localization, but for direct double-strand breaks (IR), FA core complex is not needed.

The FA core complex may physically interact with ATR-ATRIP kinase

Given the above data, we hypothesized that ATRIP may interact with the FA core complex. To test this idea, we carried out yeast two-hybrid interaction assay between ATRIP and various FA and FA-related proteins (including FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, UBE2T, FAAP24 or FAAP100) (Figure 3A). We could detect positive ATRIP interaction only with FANCL or FAAP100.

To determine whether ATRIP could interact with FA core complex components in cells, we co-overexpressed ATRIP with FANCL or FAAP100. Only FANCL co-immunoprecipitated with ATRIP in this setting (Figure 3B). These results suggest that FANCL might be the core complex subunit that associates with ATRIP protein.

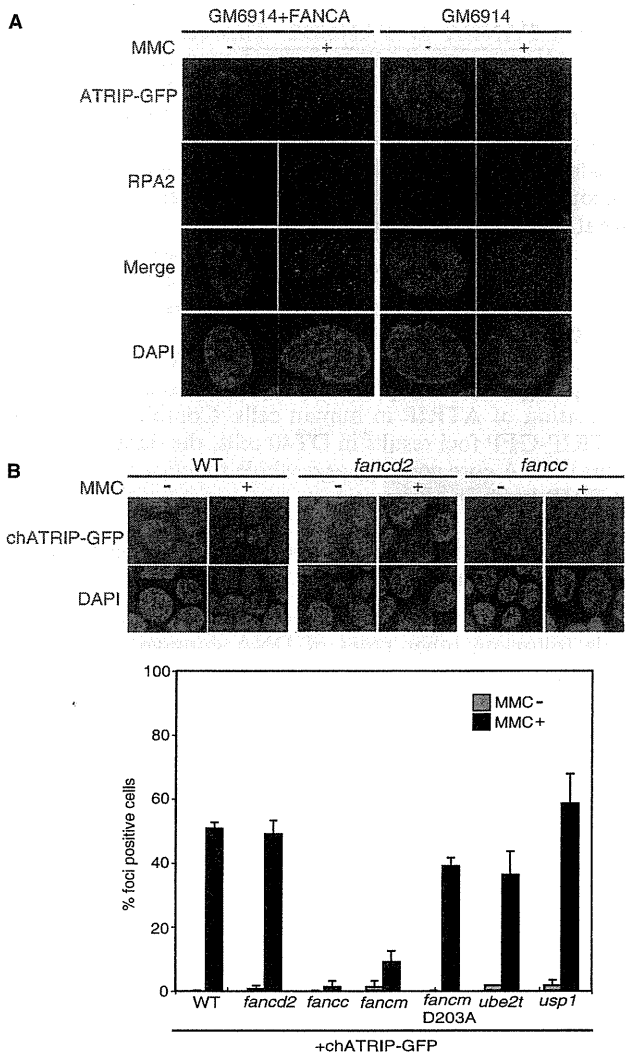

Figure 1. Defective ATRIP-GFP foci formation in FA cells. (**A**) MMC-induced human ATRIP-GFP foci formation in human FA-A (GM6914) and complemented control cells. Following transient transfection of the ATRIP-GFP expression vector, ~10% of the cells became GFP-positive 24 h after transfection. Then the cells were treated with or without MMC (100 ng/ml for 24 h), fixed and stained with anti-RPA2 antibody, and the GFP was visualized directly. The images were captured by confocal laser scanning microscopy. (**B**) MMC-induced chicken ATRIP-GFP foci in chicken DT40 mutant cells. Indicated DT40 WT and FA mutant cells were stably transfected with chicken ATRIP-GFP expression vector. Clones selected on the basis of similar GFP expression levels were subjected to analysis. Following MMC treatment (500 ng/ml for 6 h), cells were fixed and observed by confocal laser scanning microscopy. The bar graph represents mean and SD of % GFP-foci positive cells in three independent experiments. Fifty nuclei were scored in each case, and nuclei containing more than four bright GFP foci were defined as foci positive.

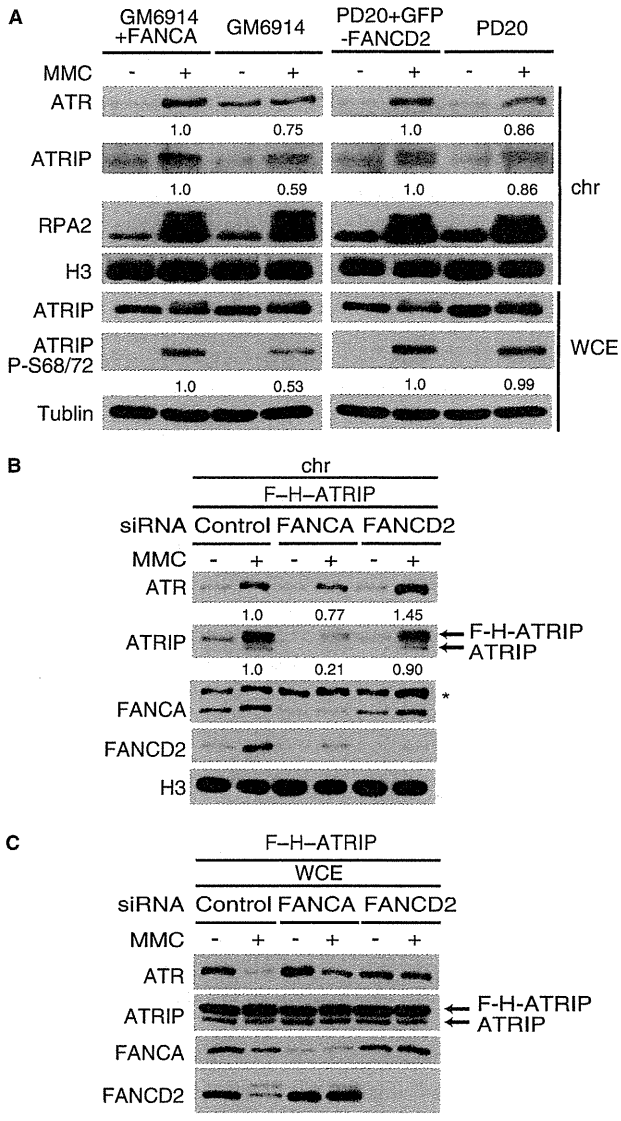

Figure 2. Chromatin localization of ATRIP is defective in human cells lacking the FA core complex. (**A**) FA-A (GM6914 fibroblast) or FA-D2 (PD20 fibroblast) and respective complemented control cells were treated with MMC (100 ng/ml for 24 h). The whole cell extract (WCE) and the chromatin fraction (chr) were prepared and analyzed by western blotting. (**B** and **C**) HeLa S3 cells expressing FLAG-HA-ATRIP (F-H-ATRIP) were transfected with siRNAs targeting FANCA or FANCD2. After 48 h, the cells were stimulated with MMC (100 ng/ml for 24 h). Chromatin fraction (B) or WCE (C) were analyzed by western blotting. The numbers indicate the ratio of the band intensity normalized to the control lane.

The FA core complex promotes binding of ATRIP to RPA-coated ssDNA

To gain further insight into the role of the core complex in the ATRIP recruitment to RPA-coated ssDNA, we assessed the amount of ATRIP protein that could be recovered from cell extracts following binding to RPA-coated ssDNA (Figure 3C). In the extracts lacking the core complex, significantly less ATRIP protein was pulled down compared with the matched control cell extracts (indicated as +A). Interestingly, the core complex components such as FANCA could be recovered from control cell extract by ssDNA beads without exogenous RPA complex. These data suggest that the core complex bound to RPA-coated ssDNA may facilitate or

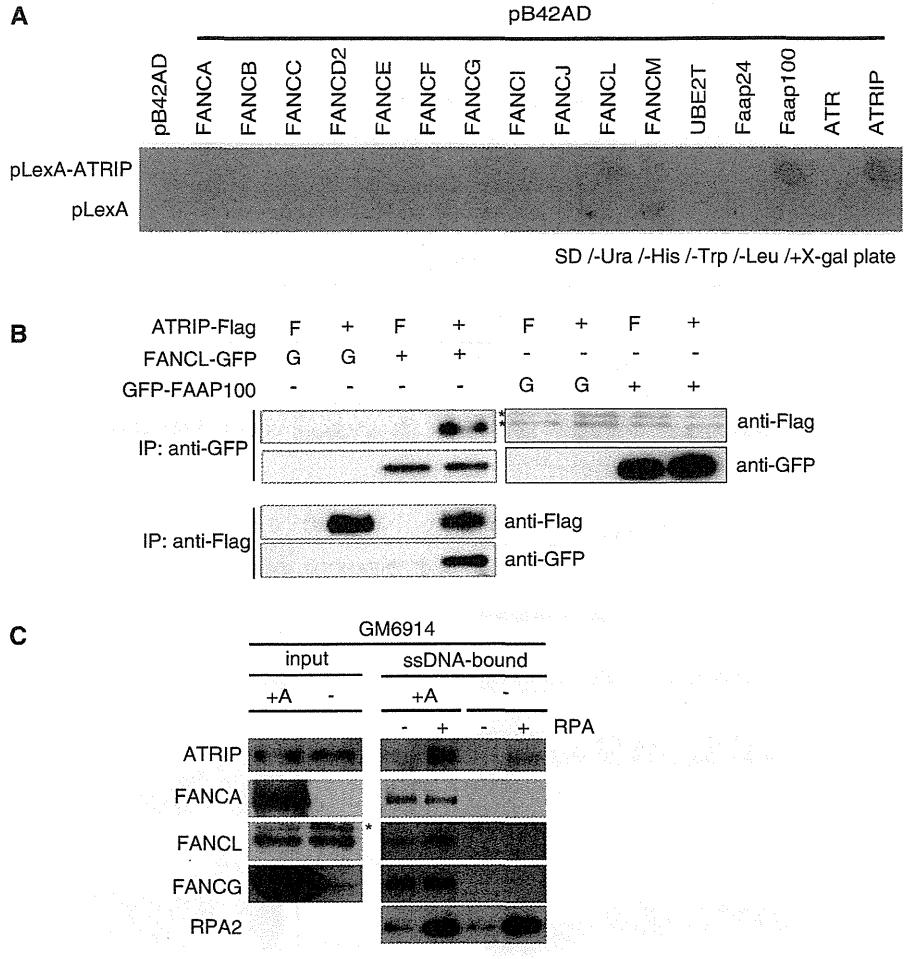

Figure 3. Interplay between FA proteins and ATRIP. (**A**) Yeast two-hybrid interaction assay between ATRIP and various full-length FA or FA-related proteins. Selected yeast transformants were spotted onto SD/-Ura/-His/-Trp/-Leu/+X-gal plates at 30° for 96 h. ATRIP–ATRIP interaction served as positive control. (**B**) Co-immunoprecipitation assay between overexpressed human FANCL-GFP or GFP-FAAP100 and ATRIP-Flag in 293T cells. F or G, Flag or GFP vector without insert. (**C**) RPA-ssDNA pull-down assay from extracts of FA-A (GM6914) and complemented FA-A cells (+A). ssDNA-attached magnetic beads preincubated with or without recombinant RPA were mixed with cell extracts. After washing, the bound material was analyzed by western blotting.

stabilize ATRIP binding to RPA through either direct or indirect interaction with ATRIP.

Loss of FANCI phosphorylation in cells lacking the FA core components

We wished to determine how the deficiency in the FA core complex affects signaling events through ATR because the altered ATRIP-ATR chromatin localization should have a consequence on downstream signaling events. Although mild S-phase checkpoint defects in FA cell lines have been reported (42–44), MMC-induced CHK1 phosphorylation was not significantly affected in cells deficient in the FA core components (Figure 4A, C and D). Interestingly, previous reports have indicated that CHK1 phosphorylation can occur without ATRIP focus formation, in cells harboring an ATRIP N-terminal deletion (45,46) or an LG mutant (47), which is incapable of binding to RPA or is deficient in dimerization (37,48). Further, stimulation of ATR activity by TOPBP1 can occur in the absence of RPA binding to ATRIP (49). Thus, an association of a residual amount of ATRIP-ATR kinase with TOPBP1 in chromatin might be sufficient to phosphorylate CHK1.

Next, we investigated FANCI phosphorylation in DT40 mutants because FANCI is the critical ATR substrate in the FA pathway (20) following DNA damage. We used the Phos-tag western blotting (17) to detect phosphorylated FANCI. Phospho-proteins could be detected as slower migrating bands in a Phos-tag-containing polyacrylamide gel electrophoresis gel. *FANCI* K525R knock-in DT40 cells were used as a control, in which the FANCI monoubiquitination site (Lysine) was replaced with Arginine by gene targeting (Supplementary Figure S4). This mutant was defective in FANCI monoubiquitination, similarly to *fancl, fancc* and *fancd2* mutants, yet FANCI phosphorylation was clearly observed (Figure 4B).

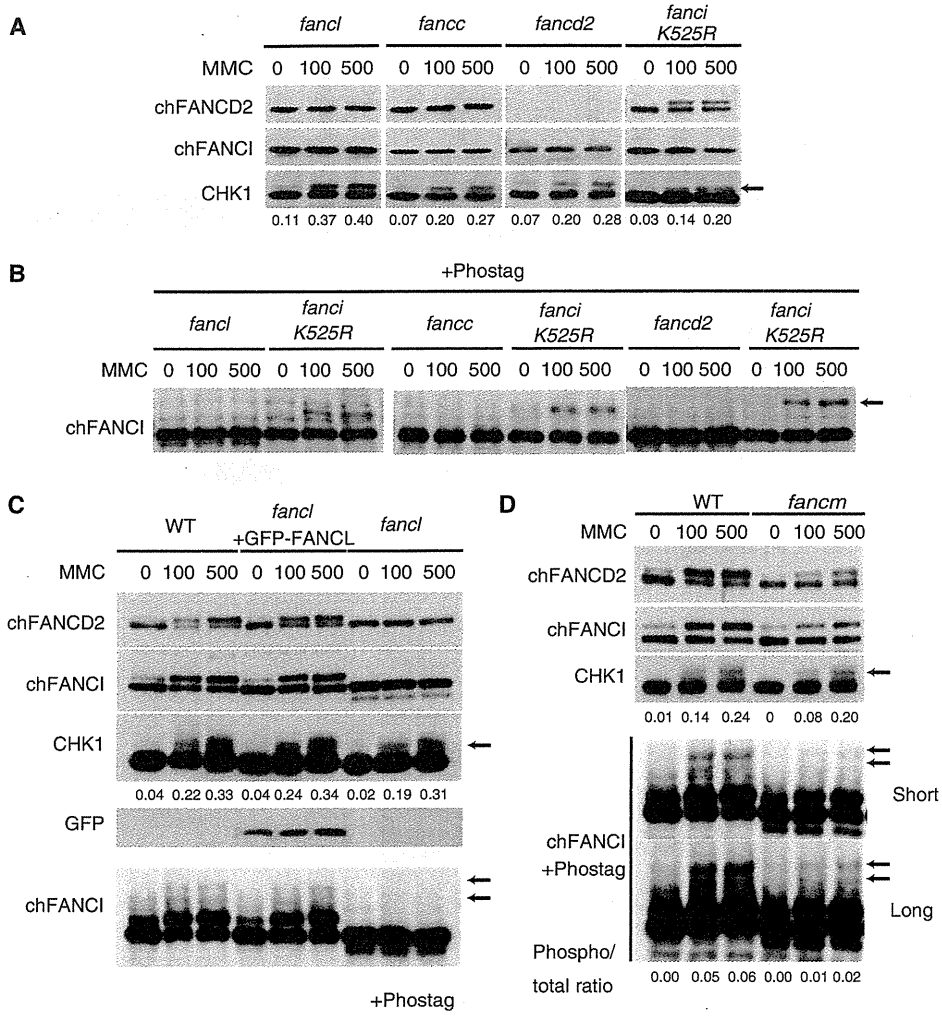

Figure 4. FANCI phosphorylation mediated by ATR is defective in cells lacking FANCD2 or the FA core complex. (**A**) Cells were treated with MMC (100 or 500 ng/ml for 6 h) and analyzed with western blotting. (**B**) Samples were prepared as in A, and analyzed with Phos-tag western blotting. (**C**) Phos-tag western blotting of FancI protein in *fancl* cells and cells complemented with GFP-chFANCL. (**D**) WT and *fancm* cells were stimulated as indicated and analyzed by western blotting with or without Phos-tag. Numbers indicate ratio of phosphorylated CHK1 or FANCI to total CHK1 or FANCI, respectively.

Strikingly, *fancl, fancc* and *fancd2* mutant cells were completely deficient in FANCI phosphorylation (Figure 4B). Furthermore, *fancl* cells complemented with GFP-chFANCL (27) could phosphorylate FANCI at a level comparable with WT cells (Figure 4C). *fancm* cells showed an intermediate phenotype, with FANCI phosphorylation ~20–30% of normal observed following MMC treatment (Figure 4D). This is consistent with the weakened but still clearly detectable FANCD2 monoubiquitination in *fancm* cells (Figure 4D). A stimulatory role of *FANCM* in FANCD2 monoubiquitination has been previously described (29,50–52).

FA pathway activation is independent of RAD17 or TOPBP1

The results described above and our previous studies (17,20) clearly indicated that FANCI phosphorylation is mediated by ATR kinase in a manner dependent on the FA core complex and FANCD2 protein. This phosphorylation event then triggers FA pathway activation, leading to FANCD2 monoubiquitination and DNA repair. It has been well accepted that the phosphorylation of the ATR substrates requires the kinase activator TOPBP1 as well as its recruiter, the 9-1-1 complex, which is loaded by the RAD17-RFC complex (21–23). To gain more insight into the interplay between ATR and the FA pathway, we wished to test whether the activation of the FA pathway is affected by the absence of the canonical ATR activators RAD17 or TOPBP1. Surprisingly, MMC-induced FANCD2 and FANCI monoubiquitination were only slightly affected by the loss of RAD17 gene (Figure 5A), while CHK1 phosphorylation was severely diminished in the knockout DT40 cell lines (Figure 5A). Consistently, FANCI phosphorylation was

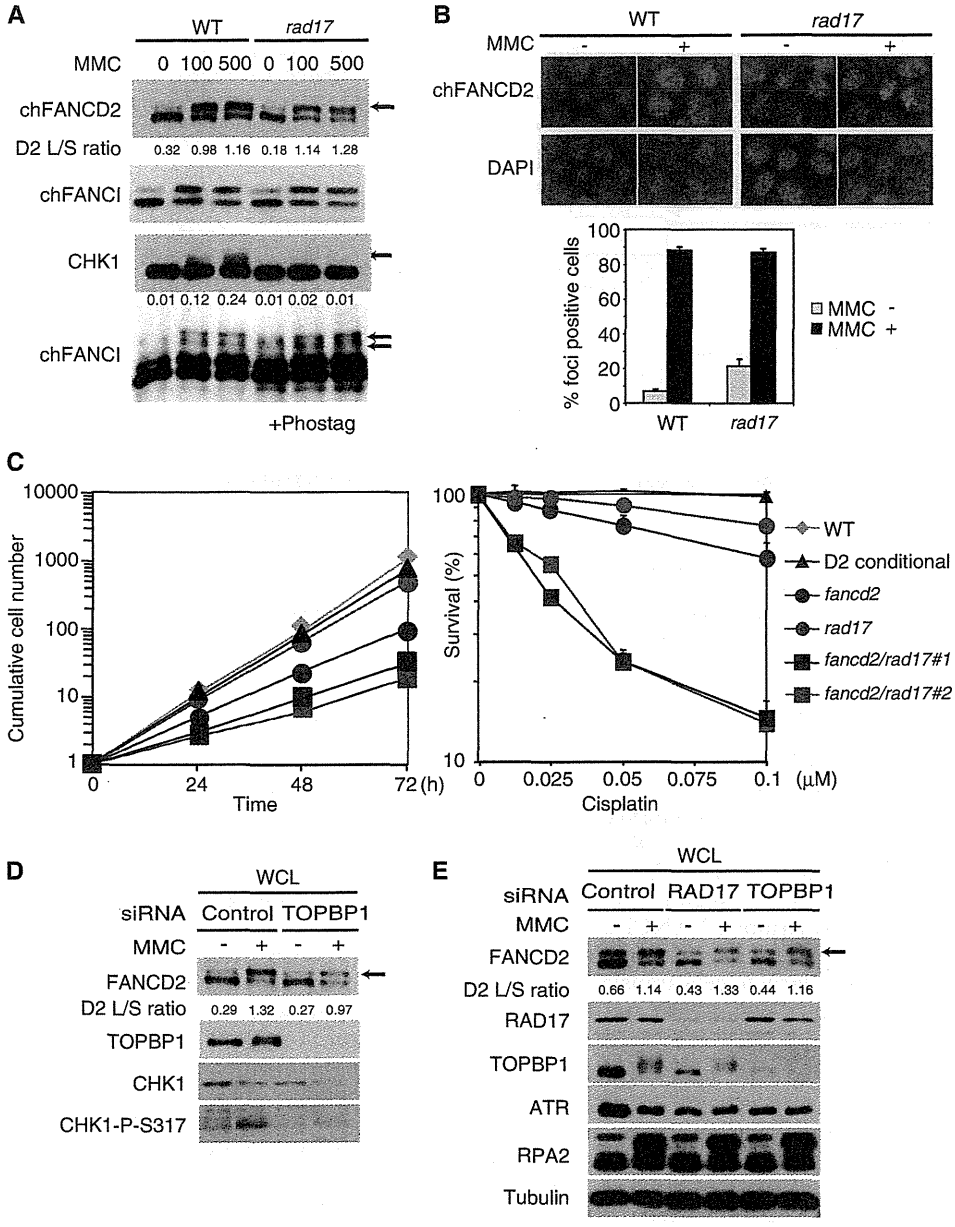

Figure 5. RAD17 and TOPBP1 are dispensable for FA pathway activation. (**A**) Cells were treated with MMC (100 or 500 ng/ml for 6 h) and analyzed with western blotting with or without Phos-tag. Numbers indicate ratio of phosphorylated CHK1 to total CHK1. (**B**) FANCD2 foci formation following MMC treatment (500 ng/ml for 6 h) was analyzed in DT40 WT and *rad17* mutant cells by fluorescent microscopy. The bar graph represents mean and SD of % FANCD2-foci positive cells in three independent experiments. Fifty nuclei were scored in each case, and nuclei containing more than four bright foci were defined as foci positive. (**C**) Cell growth rate (left panel) and % survival of the cells cultured for 48 h in the medium containing cisplatin (right panel). Cell number or cell viability was assessed by flow cytometry using plastic microbeads or PI staining, respectively. The error bar represents SD in three independent experiments. (**D** and **E**) HeLa S3 cells (D) or A549 cells expressing ATRIP-GFP (E) were transfected with indicated siRNAs. After 48 h, cells were treated with MMC (100 ng/ml) for additional 24 h. WCE was analyzed by western blotting.

not defective in *rad17* cells (Figure 5A). FANCD2 focus formation was also not affected (Figure 5B). These data indicate that the FA pathway activation occurs independently of RAD17.

To verify this conclusion by a genetic test, we generated a double knockout DT40 cell line lacking both *RAD17* and *FANCD2* genes (Supplementary Figure S5). The double knockout cells grew slower and were much more sensitive to cisplatin compared with either single mutant cell line (Figure 5C), suggesting a synergistic relationship. We also used siRNA knockdown in HeLa S3 or A549 cells expressing ATRIP-GFP. FANCD2 monoubiquitination

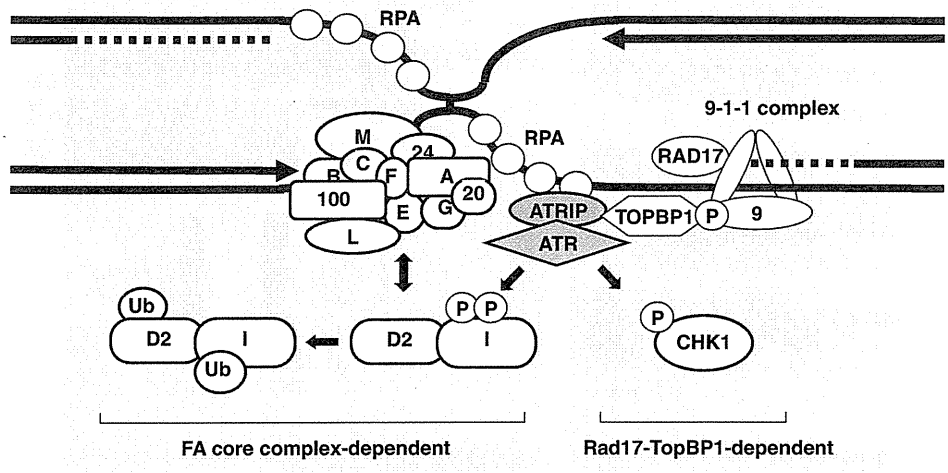

Figure 6. Our proposed model for two distinct pathways in ICL-induced ATR signaling. RPA complex accumulates on exposed ssDNA region surrounding the ICL (middle). RPA-ATRIP binding, which is facilitated by the FA core complex, promotes ATR recruitment. In the left side of the pathway, which is dependent on the FA core complex but not on the RAD17-TOPBP1 pathway, FANCI is phosphorylated, triggering FANCD2 monoubiquitination. In the right side, CHK1 is phosphorylated depending on the RAD17-TOPBP1 pathway, but the FA core complex is largely dispensable for the CHK1 activation.

was affected only slightly by siTOPBP1 treatment in HeLa S3 (L/S ratio 1.32 in control versus 0.97 in TOPBP1-depleted cells), whereas CHK1 phosphorylation was mostly abrogated (Figure 5D). In siRAD17- or siTOPBP1-treated A549 cells (Figure 5E), FANCD2 monoubiquitination was only slightly diminished compared with control. Furthermore, FANCD2 still efficiently accumulated in subnuclear foci and in chromatin following MMC damage similarly to cells transfected with control siRNA (Supplementary Figure S6A and B).

DISCUSSION

ATRIP-ATR kinase is essential for checkpoint signaling via CHK1 phosphorylation (21–23), and it is also required for DNA repair by activating the FA pathway through FANCI phosphorylation (17,20). It has been well accepted that ATR signaling proceeds in two steps: a localization step via ATRIP-RPA interaction, and then enzymatic activation by ATR-TOPBP1 interaction. In this study, we attempted to clarify interplay between ATR signaling and the FA pathway. Our data indicate that the FA core complex (including FANCM) regulates the former step, and also connects ATR and the FA pathway activation. On the other hand, the latter step is not critical for the FA pathway. There have been several reports that demonstrate roles of the FANCM/FAAP24 complex in the ATR signaling independent of the FA core complex (52–56). For example, FANCM has a role for chromatin accumulation of RPA (55,56) or TOPBP1 (54) following DNA damage. Our results complement these previous studies and indicate that other components of the FA core complex have a role in the ATR signaling.

In the current study, we provide evidence that the FA core complex affects the localization step by facilitating ATRIP interaction with RPA-ssDNA. Of note, FANCL has been reported to play an upstream role in RPA-ATR-Chk1 signaling in Xenopus egg extracts stimulated by plasmids carrying an ICL (57). The present results extend these studies to deletion of specific components of the FA core complex in mammalian and avian somatic cells, and reveal that FA core complex defects are associated with impaired intracellular and chromatin localization of ATRIP.

The mechanisms for enhancing ATRIP-RPA interaction remain unknown. ATRIP-FANCL interaction was detected using yeast two-hybrid assay and by co-immunoprecipitation of the transfected proteins. However, whether the endogenous proteins actually interact is currently unclear. Because the E2 enzyme UBE2T is not required for this process, the FA core complex stabilizes the interaction independently of the ubiquitination activity. The ATPase activity of FANCM is also dispensable. A previous study indicated that *in vitro* binding of ATRIP-ATR to RPA-ssDNA is enhanced by an unknown human protein (37). It will be interesting to test whether this unknown factor is a component of the FA core complex.

We showed that, in cells lacking the FA core complex, phosphorylation of ATRIP as well as FANCI protein is defective. MMC-induced CHK1 phosphorylation occurs normally in FA knockout cells. Loss of FANCI phosphorylation is functionally important because it is the triggering event in activation of the FA pathway (17). However, it is obscure whether defective ATRIP-ATR chromatin localization is the direct cause of the loss of FANCI phosphorylation. The FA core complex may function

downstream of ATRIP-ATR as an essential link to connect FANCI with ATR, resulting in FANCI phosphorylation. It is possible that ATR phosphorylates FANCI when it is in a complex with FANCD2. We have previously reported that efficiency of FANCI phosphorylation by ATR *in vitro* is enhanced by the presence of FANCD2 (20). Interestingly, a recent report suggests that chromatin binding of FANCI depends on FANCD2 in Xenopus egg extracts (58). Collectively, these results indicate the defective core complex abolishes the upstream phosphorylation events on FANCI in addition to its ubiquitin E3 ligase activity, thus blocking the FA pathway signaling at two levels.

Our data also indicate that TOPBP1 and RAD17 do not play a major role in FA pathway activation. This is consistent with our previous observation that ATRIP does not require TOPBP1 interaction to activate the FA pathway (20). An *in vitro* study has shown that at high concentration of substrate the influence of TOPBP1 on ATR kinase activity is less pronounced (59). In FA pathway activation, the phosphorylation substrate FANCI may accumulate in damaged chromatin, perhaps though the interaction with FANCD2 and/or the core complex (60,61), achieving concentrations that reduce the requirement for TOPBP1. Alternatively, the locally accumulated ATRIP-ATR might have sufficient activity to phosphorylate FANCI without TOPBP1 stimulation. Interestingly, auto-phosphorylation of ATR on Thr 1989 is not dependent on the presence of TOPBP1 (62).

Given these findings, we propose two distinct subpathways in ATR signaling (Figure 6). One is dependent on RAD17-TOPBP1 pathway in which the important substrate is CHK1. This pathway is also affected by the FANCM-FAAP24 subcomplex. The other pathway is dependent on the FA core complex, and critical for phosphorylating ATRIP, FANCI and perhaps FANCD2 and BLM (63). Other substrates in this subpathway of ATR signaling may be identified.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6.

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The HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin modulates radiosensitivity by downregulating serine/threonine kinase 38 via Sp1 inhibition

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Abstract The ansamycin-based HSP90 inhibitor 17-AAG (17-allylamino-17-demethoxygeldanamycin) combats tumors and has been shown to modulate cellular sensitivity to radiation, prompting researchers to use 17-AAG as a radiosensitizer. 17-AAG causes the degradation of several oncogenic and signaling proteins. We previously demonstrated that oxidative stress activates serine/threonine kinase 38 (STK38), a member of the protein kinase A (PKA)/PKG/PKC-like family. In the present study, we investigated how 17-AAG affects STK38 expression, and evaluated STK38's role in the regulation of radiosensitivity. We found that 17-AAG depleted cellular STK38 and reduced STK38's kinase activity. Importantly, 17-AAG downregulated the *stk38* gene expression. Deletion analysis and site-directed mutagenesis experiments demonstrated that Sp1 was required for the *stk38* promoter activity. Treatment with 17-AAG inhibited Sp1's binding to the *stk38* promoter by decreasing the amount of Sp1 and knocking down Sp1 reduced STK38 expression. Moreover, 17-AAG treatment or STK38 knockdown enhanced the radiosensitivity of HeLa cells. Our data provide a novel mechanism, mediated by *stk38* downregulation, by which 17-AAG radiosensitizes cells.

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1. Introduction

STK38 (serine/threonine kinase 38), also known as NDR1 (nuclear Dbf2-related 1; GenBank Accession No.: NP009202), is a serine/threonine protein kinase belonging to a subclass of the protein kinase A (PKA)/PKG/PKC-like (AGC) family,[1–3] which includes cAMP-dependent kinase, protein kinase B and protein kinase C. The STK38 family includes *Drosophila melanogaster* TRC, *Schizosaccharomyces pombe* Orb6, *Saccharomyces cerevisiae* Cbk1 and Dbf2 and mammalian STK38, STK38L/NDR2, LATS1 (large tumor suppressor 1), and LATS2.[1–3] Cbk1 and Orb6 regulate cell morphology.[4,5] Dbf2 is a cell cycle-regulated kinase required for the cycle to progress through anaphase.[6] STK38 and STK38L, which are broadly expressed in the mouse brain,[7] contribute to dendritic spine development and excitatory synaptic function.[8] STK38's activity is regulated by MST3 (mammalian sterile 20-like 3),[9] the cofactors MOB1 (Mps one binder 1) and MOB2,[10,11] or GSK-3 (glycogen synthase kinase-3).[12] We previously demonstrated that STK38 is involved in regulating MAPK (mitogen-activated protein kinase) signaling pathways and the oxidative stress response.[12,13]

Heat shock proteins (HSPs), a major class of molecular chaperones, play vital roles in cellular stress responses and cancer.[14] One particular chaperone, HSP90, dynamically promotes the conformational maturation of its client proteins and protects them from degradation by assembling client-HSP90 complexes using the chaperone machinery.[15,16] HSP90 is of considerable interest in the search for new therapeutic cancer targets, since most HSP90 client proteins are oncogenic proteins and protein kinases that regulate cell survival, proliferation, invasion, metastasis and angiogenesis.[14,17] The natural products radicicol and geldanamycin, along with their derivatives, combat tumors by inhibiting HSP90's intrinsic ATPase activity, causing HSP90's client proteins to be degraded via the ubiquitin–proteasome pathway.[18,19] The geldanamycin analogue 17-AAG (17-allylamino-17-demethoxygeldanamycin), a benzoquinone ansamycin, has similar anti-tumor properties and fewer associated side-effects.[20] In tumor cells, HSP90 is present in multi-chaperone complexes that have high ATPase activity and a strong binding affinity for 17-AAG. Since this is not the case in normal cells, 17-AAG is selective for tumors.[21] Studies have also shown that 17-AAG can radiosensitize tumor cells.[22,23]

Here we show that 17-AAG downregulates STK38 by inhibiting Sp1, and provide evidence that STK38 is a key factor in cellular sensitivity to ionizing radiation.

2. Materials and methods

2.1. Cell culture and stimulation

HeLa cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Osaka). HCT116 cells were obtained from American Type Culture Collection (Manassas, VA). HEK293T and MCF-7 cells were gifts from Dr. Katsuji Yoshioka (Kanazawa University) and Dr. Kazuya Hirano (Tokyo University of Pharmacy and Life Sciences), respectively. HeLa, HEK293T, and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium/F-12 (1:1) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT) and 1% penicillin/streptomycin (Sigma). HCT116 cells were cultured in McCoy's 5A (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were treated with either dimethyl sulfoxide (DMSO) (Sigma) or 17-AAG (Sigma) for 2–16 h. For the combined treatment, HeLa cells were treated with either DMSO or 17-AAG for 12 h, X-ray-irradiated, incubated for an additional 2 h, harvested and assayed. MG132 (Sigma) and *clasto*-Lactacystin β-lactone (Calbiochem, Darmstadt, Germany) were stored as 10 mM stock solutions in DMSO and used at 10 μM. Cells were irradiated with an X-ray generator (Pantak HF 350, Shimadzu, Kyoto) operating at 200 kV–20 mA with a filter of 0.5 mm Cu and 1 mm Al at a dose rate of 1.46 Gy/min; 46 cm FSD.

2.2. Western blot analysis

Western blot analysis was performed as described previously.[13] Equivalent amounts of total cell lysates were separated by SDS–PAGE. Proteins separated in the gel were transblotted onto Immobilon PVDF membranes (Millipore, Bedford, MA). The membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% non-fat dry milk and incubated with anti-STK38,[13] anti-NDR1 (YJ-7, Santa Cruz Biotechnology, Santa Cruz, CA), anti-C/EBP beta (GeneTex, Irvine, CA) or anti β-Actin (Sigma) antibodies. The blots were triple-washed with TBS-T and incubated with secondary peroxidase-conjugated antibodies (Dako, Glostup, Denmark). Signals were detected on X-ray films (GE Healthcare, Buckinghamshire, UK) using an enhanced chemiluminescence detection system (GE Healthcare). STK38 was quantified using an LAS-1000 mini luminescent image analyzer (Fuji Film, Tokyo).

2.3. Immune-complex kinase assays

Cell lysates were prepared as described above, incubated with specific antibodies and mixed with protein A/G agarose. Immune-complex kinase assays were performed as described previously.[13]

2.4. Semi-quantitative RT-PCR analysis

Total RNA was isolated from cells treated with DMSO or 17-AAG using the Ultraspec RNA Isolation System (Biotecx Lab, Inc., Houston, TX). RNAs were

converted to cDNA using SuperScript III reverse transcriptase (Invitrogen Carlsbad, CA) and oligo(dT)$_{12-18}$ (GE Healthcare), and the cDNAs were amplified by polymerase chain reaction (PCR) using Pfx DNA polymerase (Invitrogen) and the following primer sequences:

stk38 (forward): ATGGCAATGACAGGCTCAAC ACCTTGCTC
stk38 (reverse): GCCTACTGTGGAGAAGGCTAG CTGACG.

Primers for β-actin were purchased from Stratagene (La Jolla, CA). PCR products were analyzed by electrophoresis on 2% agarose gels.

2.5. Plasmids

The *stk38* promoter (nt −877 to −11) was cloned by PCR using genomic DNA from HeLa cells as a template. Kpn I and Xho I sites were introduced into the forward and reverse primers, respectively, for cloning convenience. The PCR-amplified *stk38* fragment was digested with Kpn I and Xho I and ligated into the pGL3-Basic plasmid (Promega, Madison, WI). The 5′-region of the promoter was deleted by PCR. PCR fragments were subcloned into the pGL3-Basic plasmid as described above. Sp1-mutant constructs were generated using the GeneArt site-directed mutagenesis system (Invitrogen). The following mutated Sp1 oligonucleotide sequences were used:

Sp1 single-mutant forward: 5′-GGGGGTGAAGGGA GGGGCAGTTCGGGCCACGCAAGCGCAGT-3′
Sp1 single-mutant reverse: 5′-ACTGCGCTTGCGT GGCCCGAACTGCCCCTCCCTTCACCCCC-3′
Sp1 double-mutant forward: 5′-GCCCTAGGCA GGGGGTGAAGTTAGGGGCAG-3′
Sp1 double-mutant reverse: 5′-CCCGAACTGCCC CTAACTTCACCCCCTGCC-3′.

The mammalian STK38 short hairpin (sh) RNA expression vector was described previously.[13] All constructs were confirmed by sequencing.

2.6. Reporter assay

HeLa cells were plated onto 24-well plates at 1×10^4 cells/well, 1 day prior to transfection. The cells were transfected using FuGENE HD (Roche, Indianapolis, IN) with 50 ng pRL (*Renilla* luciferase)-SV40 and 1.0 μg pGL3 (*Firefly* luciferase) reporter plasmids containing the *stk38* promoter. Twenty-four hours after transfection, cell extracts were prepared and luciferase activity was measured, as described by the manufacturer of the reporter system (Promega). The luciferase activity was measured using a luminescencer (AB-2200; ATTO, Tokyo).

2.7. Cell-viability and colony-formation assays

To assay cell viability, HeLa cells were transiently transfected with either a non-targeting or an *stk38*-specific shRNA expression vector using FuGENE HD, double-washed 24 h later with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS), and cultured for 48 h in medium containing 2 μg/ml puromycin (Invivogen, San Diego, CA). The cells were washed twice with PBS, left untreated or X-ray-irradiated at 3 Gy in the absence or presence of 17-AAG, and assayed for cell viability 48 h later by staining with Annexin V-FITC and propidium iodide (PI) using a MEBCYTO Apoptosis kit (MBL, Nagoya). Cell death was defined as the total percentage of cells positive for PI, Annexin V or both. All samples were counted, and more than 5000 cells were analyzed for each condition using a flow cytometer (EPICS XL System II, Beckman Coulter, Brea, CA).

Cell survival was measured by a colony-formation assay. HeLa cells were pretreated with 17-AAG or DMSO for 12 h, then X-ray-irradiated (0–5 Gy), trypsinized, diluted, counted and seeded into 60-mm dishes at various cell densities. After 7 days, the colonies were stained with crystal violet, and those containing more than 50 cells were counted. To determine the survival of STK38-knockdown cells, HeLa cells were transiently transfected with either a non-targeting or an *stk38*-specific shRNA expression vector, then selected with puromycin as described above, and assayed by colony formation. The X-ray dose-survival curves were fitted to a linear-quadratic (LQ) equation. The radiation enhancement ratio (RER) for 17-AAG or *stk38* knockdown was determined using the surviving fraction $SF_{0.5}$, determined from the clonogenic assay.

2.8. Preparation of nuclear extracts and gel-shift analysis

To prepare nuclear extracts, 1×10^7 HeLa cells were washed with PBS and suspended in 200 μl of hypotonic buffer A (10 mM Hepes–KOH, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.4% NP-40, and 1 mM DTT) containing a protease inhibitor cocktail (Nacalai tesque). The suspension was incubated on ice for 10 min and then centrifuged at 1000*g* for 5 min to obtain nuclear pellets. The pellets were washed twice in ice-cold buffer A and resuspended in 100 μl of buffer B (20 mM Hepes–KOH, pH 7.9, 0.4 M NaCl, 2 mM EDTA, and 1 mM DTT) containing protease inhibitors. The suspension was incubated for 30 min on ice and then spun at 20,000*g* for 20 min. The supernatants were dialyzed against buffer C (20 mM Hepes–KOH pH 7.9, 50 mM KCl, and 1 mM DTT) containing protease inhibitors. Gel-shift assays were performed in a 10 μl final volume containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, and 0.25 μg of poly (dI–dC). Binding reactions were carried out

using 5.0 μg of nuclear extract and 1 μl of [^{32}P] dCTP-labeled probe at room temperature for 20 min. For competition assays, 10 μM unlabeled Sp1 consensus oligonucleotides (Promega) was added to the reaction mixture. For super-shift experiments, 1 μg of anti-Sp1 antibody (H-225, Santa Cruz Biotechnology) was added to the reaction mixture 10 min before adding the ^{32}P-labeled probe. The final reaction mixture was separated on a 5% non-denaturing polyacrylamide gel in 0.5× TBE at 350 V for 20 min. The following *stk38* promoter-specific oligonucleotides were used in the Sp1 gel-shift analysis:

*stk*38 wild-type: 5′-GGGGGTGAAGGGAGGGG-CAGGGCGGGGCCA-3′
*stk*38 single-mutant (sm): 5′- GGGGGTGAAGGG AGGGGCAGTTCGGGGCCA-3′
*stk*38 double-mutant (dm): 5′- GGGGGTGAAG TTAGGGGCAGTTCGGGGCCA-3′.

2.9. ChIP assay

For chromatin immunoprecipitation (ChIP) assays, 1×10^7 HeLa cells were treated with either DMSO or 17-AAG and fixed with 1.0% formaldehyde for 8 min at room temperature. The fixed cells were washed twice with ice-cold PBS and harvested with ice-cold PBS containing 1 mM PMSF. The cell pellets were resuspended in cell lysis buffer [50 mM Tris–HCl (pH 8.1), 1% SDS, 10 mM EDTA] containing protease inhibitor cocktail, and lysed on ice for 30 min. To shear chromatin DNA into 200–1000-bp lengths, the lysates were sonicated with five sets of 10-s pulses using a Branson Sonifier 150 W SLPe at 12% of maximum power. The sonicated lysates were then spun at 10,000*g* for 20 min. After measuring the DNA concentration of the sheared chromatin in the supernatants, the sheared chromatin was separated by electrophoresis, stained with ethidium bromide and visualized by UV emission. ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions, as follows: 2 μg of the sheared chromatin was immunoprecipitated with 1 μg of anti-Sp1 or anti-RNA polymerase II antibody (H-224, Santa Cruz Biotechnology) in ChIP dilution buffer (16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, and 1.2 mM EDTA) containing a protease inhibitor cocktail. Sp1-associated or RNA polymerase II-associated DNA was amplified by PCR using the following promoter-specific primers:

stk38 (forward): 5′-ATGGTACCGAGGTAAGCT GGGTGGGTGATG-3′
stk38 (reverse): 5′-AAACTCGAGCGCGACTTCCC GGAGCGGCCG-3′
gapdh (forward): 5′-TACTAGCGGTTTTACGG GCG-3′
gapdh (reverse): 5′-TCGAACAGGAGGAGCAGA-GAGCGA-3′.

PCR products were separated by electrophoresis through a 2% agarose gel, stained with ethidium bromide and visualized by UV emission.

3. Results

3.1. 17-AAG treatment reduces STK38 protein levels

We previously demonstrated that oxidative stress stimulates STK38, and that STK38 activation is required to protect cells from oxidative stress.[12] Those findings, along with reports that inhibiting HSP90 enhances the cellular sensitivity to oxidative stresses by degrading or downregulating signaling proteins,[14,22,23] led us to investigate whether inhibiting HSP90 affects STK38 expression. Treating cells with an HSP90 inhibitor provides a simple assay of whether a given protein depends on HSP90 activity, either directly or indirectly. Thus, we examined if the HSP90 inhibitor 17-AAG affected STK38 in HeLa cells, and found that the endogenous STK38 protein decreased according to 17-AAG dosage (Fig. 1A). A concentration of 1 μM 17-AAG, at which HSP90 is fully inhibited in various cell lines,[24] was maximally effective for depleting STK38. We next treated cells with 0.5 μM 17-AAG for various times, and found a time-dependent decrease in the STK38 level (Fig. 1B). The effect of 17-AAG on STK38 was similar in HEK293T, HCT116 and MCF7 cells (Fig. 1C). We also found that the kinase activity of immunoprecipitated endogenous STK38 was decreased by 17-AAG in a dose-dependent manner (Fig. 1D), probably because the expression level of STK38 was reduced.

3.2. Expression of the stk38 gene is downregulated by 17-AAG

The inhibition of HSP90 disturbs its client proteins by disrupting their chaperoning and targeting them for proteasomal degradation. We next investigated whether the ubiquitin–proteasome system is responsible for depleting STK38 when HSP90's activity is inhibited. Surprisingly, treating HeLa cells with proteasome inhibitors, 10 μM MG132 or 10 μM lactacystin, did not antagonize the 17-AAG-induced STK38 depletion (Fig. 2A). Since these findings suggested that STK38 is not degraded by the ubiquitin–proteasome pathway, we assessed the effect of 17-AAG on *stk38* gene expression using semi-quantitative RT-PCR. As shown in Fig. 2B and C, 17-AAG downregulated the *stk38* mRNA in a dose- and time-dependent manner.

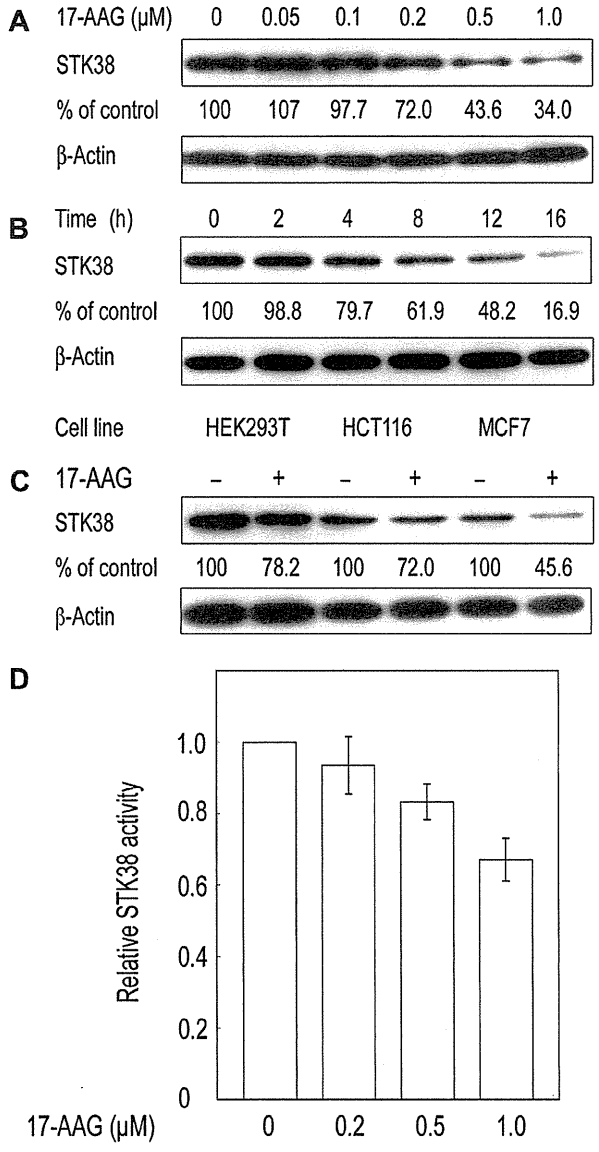

Fig. 1. Treatment with 17-allylamino-17-demethoxygeldanamycin (17-AAG) decreased serine/threonine kinase 38 (STK38) expression. (A) HeLa cells were treated with dimethyl sulfoxide (DMSO) (vehicle) or various concentrations of 17-AAG for 12 h. (B) HeLa cells were treated with 0.5 μM 17-AAG for the times indicated. (C) Various cell lines were treated with 0.5 μM 17-AAG for 12 h, then cell lysates were analyzed by Western blot with the indicated antibodies. STK38 signals were quantified and normalised against β-actin signals. (D) HeLa cells were treated with DMSO or various concentrations of 17-AAG for 12 h. STK38 kinase activity was measured in cell lysates by immune-complex kinase assay with an anti-STK38 antibody, using a synthetic peptide as the substrate. Data represent the average and standard deviations of three independent experiments, expressed as STK38 activity relative to that of DMSO-treated cells.

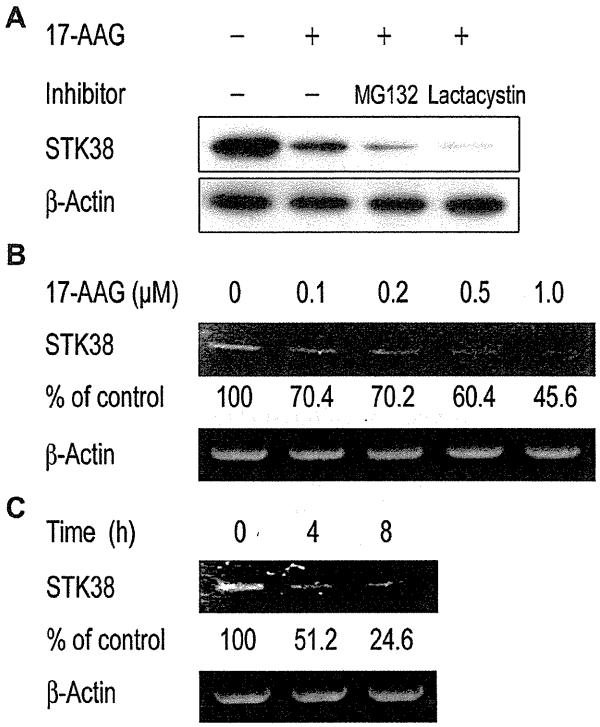

Fig. 2. Treatment with 17-allylamino-17-demethoxygeldanamycin (17-AAG) downregulated the *stk38* expression. (A) Proteasome inhibitors did not block 17-AAG-mediated serine/threonine kinase 38 (STK38) depletion. HeLa cells were treated for 12 h with 0.5 μM 17-AAG, either alone or with 10 μM MG132 or lactacystin, then cell lysates were analyzed by Western blot with the indicated antibodies. (B) HeLa cells were treated with DMSO or various concentrations of 17-AAG for 12 h. (C) HeLa cells were treated with 0.5 μM 17-AAG for the indicated times. Total RNA was prepared from treated and untreated cells, and *stk38* mRNA levels were estimated by semi-quantitative RT-PCR. STK38 signals were quantified and normalised against β-actin signals.

3.3. Sp1-dependent regulation of the stk38 promoter

To investigate how the human *stk38* gene is regulated, we constructed a luciferase reporter plasmid containing the 5′-flanking region (nt −877 to −11) of the human *stk38* gene, containing its promoter. Since computational predictive analysis of the region revealed putative consensus sequences for NF-κB, CREB, C/EBP, Sp1 and Ap1 (Fig. 3A), we applied stimuli to activate these transcriptional factors and measured their subsequent luciferase activity. However, treatment with TNF-α or Forskolin, which respectively stimulate NF-κB and CREB activity, did not significantly alter the luciferase activity driven by the *stk38* promoter (see Supplemental Fig. 1). We next constructed reporter plasmids containing 5′-serial deletions of the *stk38* promoter and measured their activity. The promoter's basal activity did not vary with deletions in the region between −877 and −280 (Fig. 3B). Deleting the region between −280 and −277 reduced the luciferase activity to 57% of that of the *stk38* promoter containing the 5′-flanking region between −877 and −11, suggesting the involvement of a transcriptional factor in the −280/−277 region. Computational analysis of this region predicted the existence of an Ap1-binding site. However, PMA, an Ap1 activator, did not stimulate the *stk38* promoter activity (Supplemental Fig. 1).

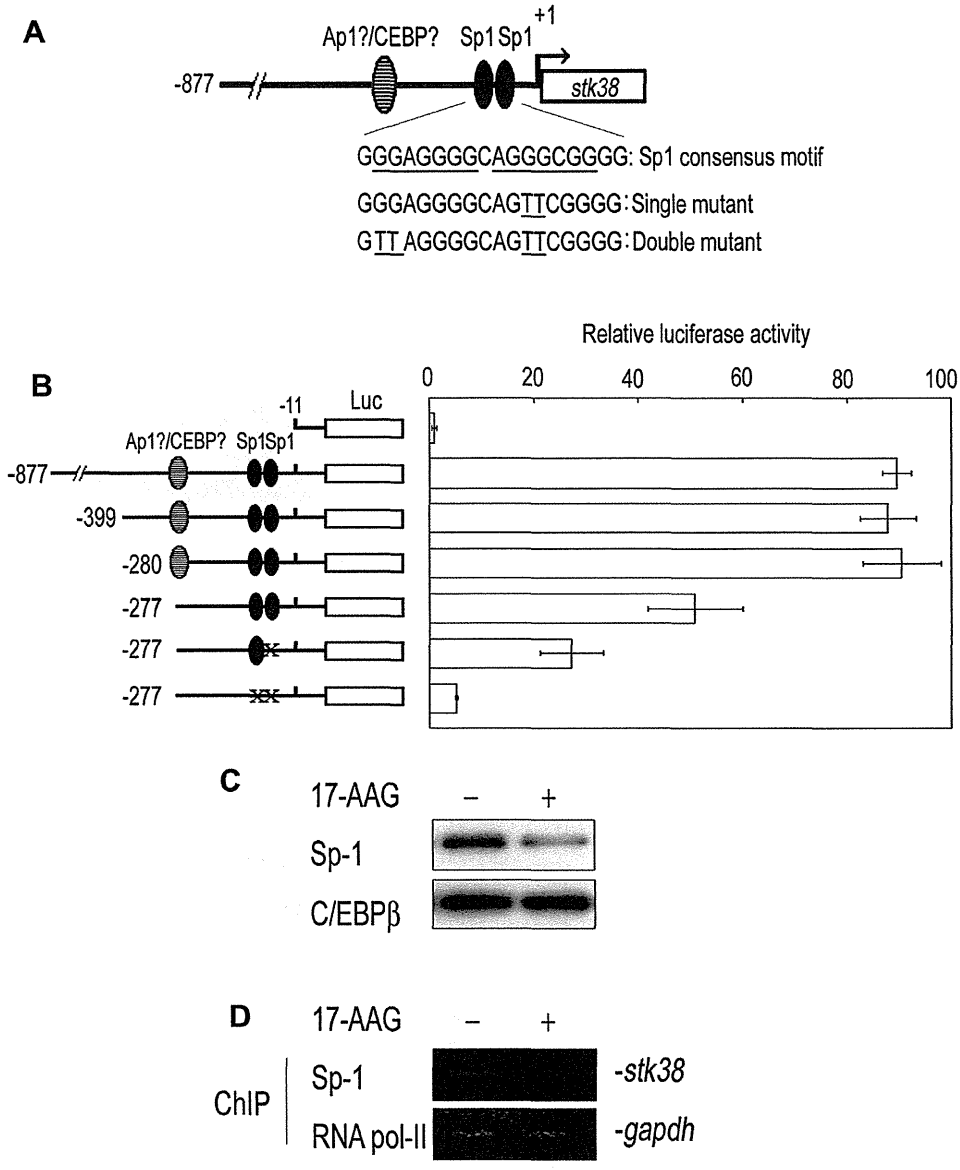

Fig. 3. Functional analysis of the *stk38* promoter. (A) Schematic representation of the human *stk38* promoter, showing putative binding sites for Sp1 transcription factors. (B) Luciferase reporter plasmids containing the human *stk38* promoter with 5′-serial deletions or a mutated Sp1-binding site were transfected into HEK293T cells; after 24 h, luciferase assays were performed on cell lysates. Data obtained from three independent experiments are expressed as the mean ± SD of *Firefly* luciferase values normalised to *Renilla*. (C) Treatment with 17-allylamino-17-demethoxygeldanamycin (17-AAG) decreased Sp1 but not C/EBPβ. HeLa cells were treated with DMSO or 1.0 μM 17-AAG for 12 h, and cell lysates were analyzed by Western blot with the indicated antibodies. (D) Sp1's DNA-binding to the *stk38* promoter is inhibited by 17-AAG. HeLa cells were treated with DMSO or 1.0 μM 17-AAG for 12 h. Sheared chromatin from HeLa cells treated with DMSO or 17-AAG was immunoprecipitated using an antibody against Sp1. The immunoprecipitated DNA was amplified by polymerase chain reaction (PCR) using specific primers targeted to the *stk38* gene promoter elements (nt −277 to −11). RNA polymerase II's DNA-binding to the *gapdh* promoter was used as an internal control.

Sequence analysis showed two Sp1 consensus binding sites in the region between −277 and −11. While site-directed mutagenesis of the Sp1 consensus site G (−63/−62) T moderately reduced the luciferase activity, mutations at both Sp1 consensus sites, G (−73/−72) T and G (−63/−62) T, decreased the luciferase activity to 5.8% of that of the *stk38* promoter containing the region between −877 and −11.

Computational analysis also indicated a putative C/EBPβ-binding site in the −280/−277 region. We next examined the effect of 17-AAG on Sp1 or C/EBPβ expression. As shown in Fig. 3C, treating HeLa cells with 1.0 μM 17-AAG reduced the level of Sp1 but not of C/EBPβ. We then investigated whether the 17-AAG-mediated degradation of Sp1 is proteasome-dependent. We found that MG132 rescued the degrada-

tion of Sp1 (Supplemental Fig. 2). Using a ChIP assay, we found that Sp1 bound to the −277/−11 region of the endogenous *stk38* promoter, and that 1 μM 17-AAG significantly inhibited this binding (Fig. 3D). Thus, Sp1's inability to bind DNA in cells treated with 17-AAG arises from the degradation of Sp1.

3.4. 17-AAG treatment inhibits Sp1-binding activity

Since our results showed that Sp1 binds the endogenous *stk38* promoter, we conducted gel-shift assays to determine whether Sp1 could bind the putative transcription factor sites in the −73/−62 region of the *stk38* promoter. A probe corresponding to the −82/−52 region of the *stk38* promoter formed four complexes, designated I–IV (Fig. 4A, lane 2); these were competed by excess amounts of unlabeled Sp1 consensus oligonucleotides (Fig. 4A, lane 3). A gel-shift assay in the presence of an Sp1-specific antibody showed that complex I was formed by Sp1, as seen from the decrease in signal intensity and the appearance of a super-shifted complex (Fig. 4A, lane 4). Mutating one putative Sp1-binding site—G (−63/−62) T, designated as sm—of the *stk38* promoter had little effect on the formation of complex I, although the mutation diminished the super-shifted complex slightly (Fig. 4A, lanes 5 and 6). Mutations at both putative Sp1-binding sites—G (−73/−72) T and G (−63/−62) T, designated as dm—completely eliminated the formation of complex I and the super-shifted complex (Fig. 4A, lanes 7 and 8). These findings were consistent with the results from the luciferase experiments (Fig. 3B). Complexes II and IV were diminished by adding excess Sp1 consensus oligonucleotides or by using a dm-mutant oligonucleotide as a probe, suggesting that the binding factors of these complexes are Sp1-like proteins. Since introducing mutations had little effect on the formation of complex III, the proteins in complex III may bind at sites other than the Sp1-binding sites. Taken together, our results indicate that Sp1 binds to the −73/−62 region of the *stk38* promoter.

We next used gel-shift assays to investigate the effect of X-ray-irradiation, either alone or in combination with 17-AAG, on Sp1's DNA-binding to the *stk38* promoter. Sp1 from X-irradiated HeLa cells had slightly lower DNA-binding activity than that from unirradiated cells (Fig. 4B, lane 3); in addition, the formation of complex IV increased, suggesting that a rearrangement of binding proteins may occur in this region. Combined treatment with X-ray-irradiation and 17-AAG significantly inhibited the formation of all of the complexes (Fig. 4B, lane 4), indicating that 17-AAG inhibited the binding activity of Sp1 and possibly Sp1-like proteins.

Since the ChIP and gel-shift experiments indicated that 17-AAG inhibited Sp1's DNA-binding activity for the *stk38* promoter, we investigated whether a reduction

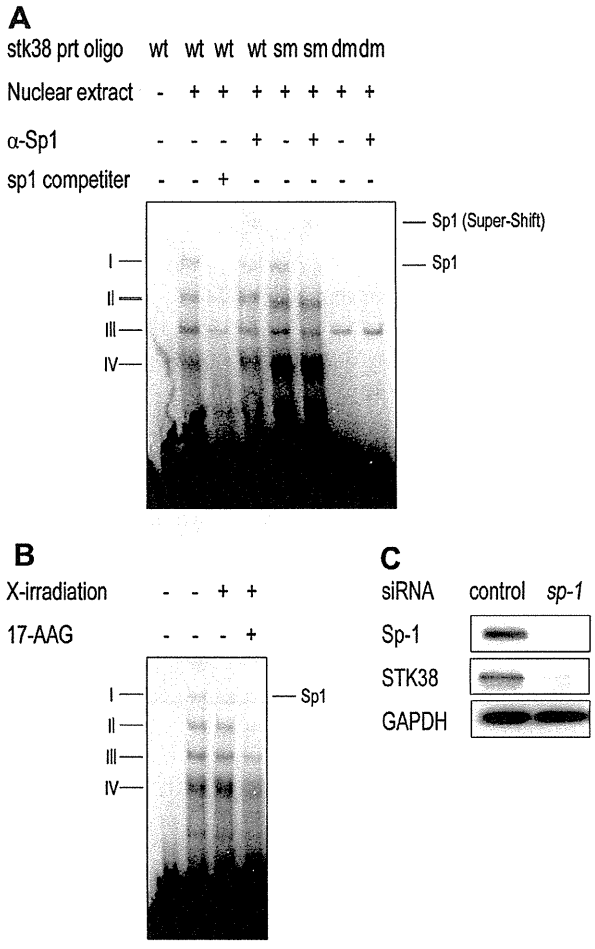

Fig. 4. Sp1's DNA-binding activity is inhibited by 17-allylamino-17-demethoxygeldanamycin (17-AAG). (A) Nuclear extracts were prepared from HeLa cells, and ^{32}P-labeled wild-type oligonucleotides encompassing the Sp1-binding sites at the region (nt −83 to −52) of the *stk38* promoter were incubated in the absence (lane 1) or presence (lane 2) of 5 μg of the nuclear extracts. Competition assays were performed in the presence of a 10× molar excess of cold Sp1 consensus oligonucleotides (lane 3). A super-shift assay was performed by incubating the reaction mixture with 1 μg of Sp1 polyclonal antibody prior to adding the radio-labeled probe (lane 4). Four DNA-protein complexes were formed (I–IV). Mutations were introduced at one or two Sp1-binding sites of the oligonucleotides: G (−63/−62) T (sm), or G (−73/−72) T and G (−63/−62) T (dm). These mutant oligonucleotides were used in gel-shift (lanes 5 and 7) or super-shift assays (lanes 6 and 8). (B) HeLa cells were pretreated with DMSO or 1 μM 17-AAG for 12 h and X-ray-irradiated (20 Gy). Nuclear extracts were prepared 2 h after irradiation. Sp1's DNA-binding activity in untreated, X-ray-irradiated, or 17-AAG-treated irradiated HeLa cells was analyzed by gel-shift assay using ^{32}P-labeled wild-type oligonucleotides. (C) Sp1 knockdown decreases serine/threonine kinase 38 (STK38) expression. HeLa cells were transfected with negative (control) or Sp1 siRNA; 48 h later, cell lysates were prepared and analyzed by Western blot using the indicated antibodies.

in Sp1 activity by *sp1* RNA interference would affect STK38 expression, and found that transfection with *sp1*-specific small interference RNA reduced the STK38 protein level (Fig. 4C). Taken together, our results suggest that 17-AAG suppresses STK38 by

inhibiting Sp1's binding to the promoter of the *stk38* gene.

3.5. 17-AAG inhibits X-ray-stimulated STK38 activity and enhances X-ray-induced cell death by promoting apoptosis

We previously reported that oxidative stress stimulates STK38 activity.[12] To further determine the effect of 17-AAG on X-ray-stimulated STK38 activity, HeLa cells were treated with 1 μM 17-AAG for 12 h and then exposed to a single dose of X-irradiation (20 Gy). Exposing HeLa cells to X-rays alone enhanced STK38's activity slightly (1.4-fold) (Fig. 5A, left panel), but did not affect its protein level. On the other hand, the combination of 17-AAG and X-ray-irradiation significantly decreased STK38's activity, probably because of the 17-AAG-mediated reduction in STK38 levels (Fig. 5A, right panel).

We next conducted colony-formation assays to investigate the impact of 17-AAG and X-ray-irradiation, singly or in combination, on cell survival. HeLa cells were treated with 0.5 μM 17-AAG for 12 h, followed by a single X-ray dose (1, 2, 3, or 5 Gy). Pretreatment with 17-AAG significantly enhanced the X-ray-induced cell death (Fig. 5B, left panel). To further evaluate the radiosensitizing effect of 17-AAG, the RER was measured using the $SF_{0.5}$ determined from the clonogenic assay.

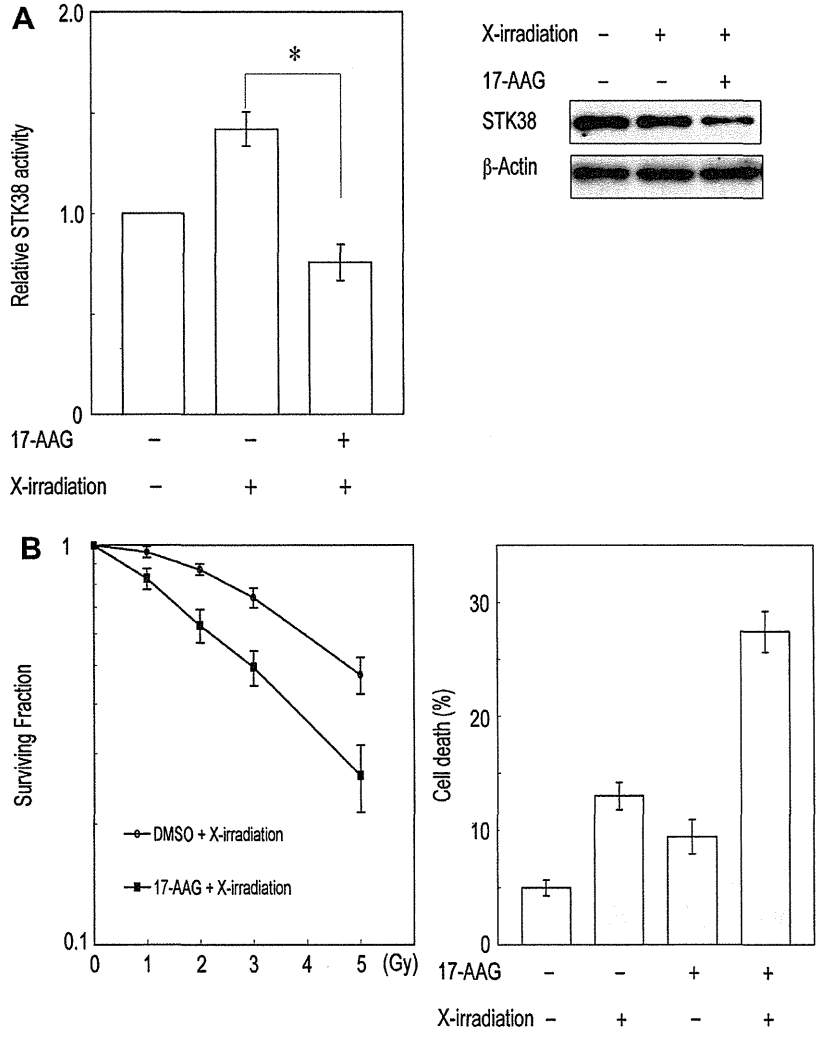

Fig. 5. Treatment with 17-allylamino-17-demethoxygeldanamycin (17-AAG) inhibited X-ray-stimulated serine/threonine kinase 38 (STK38) activity and enhanced radiosensitivity. (A) Left panel: HeLa cells were pretreated with DMSO or 1.0 μM 17-AAG for 12 h, X-ray-irradiated (20 Gy), and incubated an additional 2 h. STK38 kinase activity was measured as described in Fig. 1D. Data represent the average and standard deviations of three independent experiments, expressed as STK38 activity relative to that in cells treated with DMSO only. Statistical analysis was performed using the Student's *t*-test. Asterisks indicate statistically significant differences between X-ray-irradiation only and combined treatment with 17-AAG and X-ray-irradiation ($^*p < 0.05$). Right panel: STK38 levels were analyzed by Western blot. (B) Left panel: Clonogenic assays were performed after HeLa cells were pretreated with DMSO or 0.5 μM 17-AAG for 12 h, X-ray-irradiated at various doses, incubated an additional 2 h. Right panel: HeLa cells were pretreated with DMSO or 0.5 μM 17-AAG for 12 h and X-ray-irradiated (3 Gy); 48 h later, cell death was assessed by staining with Annexin V-FITC plus PI, using a flow cytometer.